

# Electrostatics and proton transfer in photosynthetic water oxidation

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Photosystem II (PSII) oxidizes two water molecules to yield dioxygen plus four protons. Dioxygen is released during the last out of four sequential oxidation steps of the catalytic centre ( $S_0 \Rightarrow S_1$ ,  $S_1 \Rightarrow S_2$ ,  $S_2 \Rightarrow S_3$ ,  $S_3 \Rightarrow S_4 \rightarrow S_0$ ). The release of the chemically produced protons is blurred by transient, highly variable and electrostatically triggered proton transfer at the periphery (Bohr effect). The extent of the latter transiently amounts to more than one  $H^+/e^-$  under certain conditions and this is understood in terms of electrostatics. By kinetic analyses of electron–proton transfer and electrochromism, we discriminated between Bohr-effect and chemically produced protons and arrived at a distribution of the latter over the oxidation steps of 1 : 0 : 1 : 2. During the oxidation of tyr-161 on subunit D1 ( $Y_Z$ ), its phenolic proton is not normally released into the bulk. Instead, it is shared with and confined in a hydrogen-bonded cluster. This notion is difficult to reconcile with proposed mechanisms where  $Y_Z$  acts as a hydrogen acceptor for bound water. Only in manganese (Mn) depleted PSII is the proton released into the bulk and this changes the rate of electron transfer between  $Y_Z$  and the primary donor of PSII  $P_{680}^+$  from electron to proton controlled. D1-His190, the proposed centre of the hydrogen-bonded cluster around  $Y_Z$ , is probably further remote from  $Y_Z$  than previously thought, because substitution of D1-Glu189, its direct neighbour, by Gln, Arg or Lys is without effect on the electron transfer from  $Y_Z$  to  $P_{680}^+$  (in nanoseconds) and from the Mn cluster to  $Y_Z^{ox}$ .

**Keywords:** photosystem II; water oxidation; proton release; electron transfer; electrochromism

## 1. INTRODUCTION

Oxygenic photosynthesis of green plants and cyanobacteria uses water as the source of electrons to produce carbohydrates from carbon dioxide. Two water molecules are bound at the catalytic centre at the luminal side of PSII which contains presumably four Mn atoms ( $Mn_4$ ) and a redox active tyrosine residue,  $Y_Z$ . The absorption of light oxidizes a chlorophyll *a* monomer  $P_{680}$ , to yield  $P_{680}^+$ . The latter oxidizes  $Y_Z^{ox}$  in nanoseconds which is, in turn, reduced by  $Mn_4/2H_2O$  in micro- to milliseconds. By sequential absorption of four quanta, causing one electron transfer each, the catalytic site is stepped through five increasingly oxidized states,  $S_0$  to  $S_4$ .  $S_4$  decays spontaneously to  $S_0$ , under the release of dioxygen (see Renger (2001) for a recent review). When a dark-adapted sample is excited by a series of short laser flashes, oxygen release peaks on the third flash because  $S_1$  is the most stable state in the dark.

A structural model of PSII with a 3.8 Å resolution is now available (Zouni *et al.* 2001) with a boot-shaped electron density attributable to the Mn cluster, which appears pear-shaped in another model at 3.7 Å resolution (R. J.

Shen, personal communication). The assignment of amino acids to the electron densities is still in progress. Extended X-ray absorption fine structure has revealed at least three Mn–Mn distances (2.7, 2.8 and 3.3 Å) and their minute changes as a function of the redox state (Liang *et al.* 2000; Dau *et al.* 2001; Robblee *et al.* 2001; Haumann *et al.* 2002). Still, there have remained several  $Mn_4$  topologies that are compatible with the structural data. Despite insufficient knowledge of the structure of the Mn centre, several authors have published models for the catalytic events (for recent attempts see Hoganson & Babcock 1997, 2000; Haumann & Junge 1999b; Schlodder & Witt 1999; Siegbahn & Crabtree 1999; Messinger 2000; Siegbahn 2000; Chu *et al.* 2001; Dau *et al.* 2001; Hillier & Wydrzynski 2001; Messinger *et al.* 2001; Nugent *et al.* 2001; Renger 2001; Vrettos *et al.* 2001). A remarkable feature of water oxidation is the narrow window of mid-point potentials between  $P_{680}^+/P_{680}$  (1.1–1.2 V) (Klimov *et al.* 1979; Rutherford *et al.* 1981);  $Y_Z^{ox}/Y_Z$  (1 V) (Boussac & Etienne 1982, 1984) and the Mn cluster (0.9–0.95 V for  $S_2/S_1$  and  $S_3/S_2$ ) (Vass & Styring 1991).

The net turnover of one full cycle,  $2H_2O \rightarrow 4e^- + O_2 + 4H^+$ , liberates four protons. They are not concertedly released with dioxygen, but distributed over the four redox transitions (reviewed in Lavergne & Junge 1993; Haumann & Junge 1996, 1999b). It has been noted that the internal production of protons, their retention or release into the bulk, and their shuffling back and forth between

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cofactors may provide the required 100–200 mV leeway for the catalytic centre to progress between tight energetic constraints (Krishtalik 1986, 1989; Hoganson *et al.* 1995; Mulkidjanian 1999b; Tommos & Babcock 2000).

Assaying protolytic reactions within and out of the catalytic Mn centre is complicated by the superimposition of electrostatically driven proton release into and uptake from the bulk at the periphery of PSII (Bohr effects) with the chemical proton production (by water oxidation itself). It is furthermore complicated by the lack of direct indicators for internal proton rocking. In this article, we review kinetic experiments aiming at a discrimination between ‘chemical proton production’ and ‘electrostatic proton release or uptake’ at the periphery. We describe a model for electrostatic proton per electron stoichiometries and discuss the implications for the mechanism of water oxidation.

## 2. THE VARIABLE EXTENT OF PROTON TRANSFER AS A FUNCTION OF THE FLASH NUMBER MONITORED BY ADDED pH-INDICATING DYE

Under some conditions, for example in thylakoids, PSII membranes and certain PSII core particles, the extent of proton release under excitation with short laser pulses oscillates with a period of four. The extent detected under repetitive excitation with a nanosecond-laser flash has been taken as one proton per single turnover of PSII. It has served to normalize the pattern of extents as a function of the flash number in dark adapted and thereby mainly  $S_1$ -synchronized samples. During the first few flashes, either less than one or more than one proton per PSII may be released on a certain redox transition, depending on the pH (see figure 1; Haumann & Junge 1994a; Bögershausen & Junge 1995). It is documented in figure 2 that under other conditions, for example, in oxygen-evolving PSII core particles in the presence of a detergent, the oscillations of proton release are lost and the release of one proton is detected on every redox transition, a pattern that is now independent of the pH (Renger *et al.* 1987; Lübbers *et al.* 1993; Bögershausen & Junge 1995). In the same material the oscillations can be restored by the addition of glycerol as a co-solute (Haumann *et al.* 1997b). Despite the stoichiometric pattern of proton release being highly variable as a function of the preparation, of pH and even the solvent, the pattern of oxygen evolution is often constant (Lübbers *et al.* 1993).

These observations have been interpreted in terms of a superposition of the chemical production of protons in the catalytic centre proper and of deprotonation or reprotonation of peripheral amino acids in response to transients in the centre that are electrostatically (Haumann & Junge 1996, 1999b), or perhaps conformationally triggered (Mulkidjanian 1999a).

## 3. KINETIC PROPERTIES OF PROTON TRANSFER AT THE DONOR SIDE OF PSII AS A FUNCTION OF THE FLASH NUMBER MONITORED BY ADDED pH-INDICATING DYES

Proton transfer from the donor side of PSII to added pH indicators is often biphasic. This is particularly evident

at the third flash of figure 1a. This particular trace merits some discussion. The rise time of the slow phase coincides with the rise time of oxygen release as detected by a time resolving (centrifugable) oxygen electrode. This holds true even in mutants of *Synechocystis* where the rise time of dioxygen release, concomitant with the one of the reduction of  $Y_Z^{ox}$ , is prolonged from 1.4 ms to 10 ms (Hundelt *et al.* 1998). The slowly rising phase has been attributed to chemically produced protons during the final reaction of the catalytic centre. In this way the biphasic rise has been interpreted as follows. The rapid phase represents the deprotonation of peripheral acid residues. It is electrostatically driven by the positive charge on  $Y_Z^{ox}$  and a nearby located cluster of hydrogen bonded acid or bases. This phase reverses during the electron transfer from the manganese cluster to  $Y_Z^{ox}$  that restores electroneutrality. The reversal, however, is not directly apparent because it is compensated by the synchronous appearance of the chemically produced protons. The net result is the biphasic net release as observed on giving the third flash (see figure 1a).

At another pH, namely 6.2, a slow uptake of protons follow the rapid release of a proton upon the third flash (figure 1b). How does this compare with the above interpretation? It has been interpreted as follows. At pH 6.2 the extent of electrostatically triggered proton release due to earlier flashes (e.g. the high extent at the first flash) exceeds a 1 : 1 proton per electron stoichiometry, so that the resetting of the electrostatic situation by electron donation from water calls for the re-uptake of more protons than are chemically produced upon the third flash. One may ask whether an electrostatically triggered release can exceed a stoichiometric ratio of 1 : 1 of protons over electrons abstracted from the catalytic centre? It can, indeed, as illustrated below.

The discrimination between ‘chemical’ and ‘electrostatic’ proton liberation is straightforward in the case of figure 1a (third flash). By kinetic and isotopic analyses we have found ‘chemical’ proton liberation not only upon transition of  $S_3 \Rightarrow S_4 \rightarrow S_0$  (Förster & Junge 1985; Haumann & Junge 1994a) but also on  $S_2 \Rightarrow S_3$  (Haumann *et al.* 1996; Hundelt *et al.* 1997), and possibly (but for technical reasons this is less well defined) also on  $S_0 \Rightarrow S_1$ . This has led us to a pattern of the intrinsic proton production that is 1 : 0 : 1 : 2 over the four transitions from  $S_0 \Rightarrow S_1$  to  $S_3 \Rightarrow S_4 \rightarrow S_0$  (Haumann & Junge 1999b). This is coincidental with the pattern that has been inferred from studies of electrochromic transients of chlorophylls in response to charge transients in the catalytic centre (Saygin & Witt 1985a,b; Kretschmann *et al.* 1996; Schlodder & Witt 1999).

The ‘chemical’ proton release during the transition  $S_3 \Rightarrow S_4 \rightarrow S_0$  as detected upon the third flash in figure 1a (half-rise in 1.4 ms) is kinetically distinct from its ‘electrostatic’ precursor (some 10  $\mu$ s). Less obvious to inspection by the eye but discernible by its longer rise time (some 100  $\mu$ s) and a greater kinetic H/D-isotope effect is the chemical release upon  $S_2 \Rightarrow S_3$  (Haumann *et al.* 1996; Hundelt *et al.* 1997). In both cases the comparatively slow rises coincide with the rise of the electron transfer to  $Y_Z^{ox}$ .

In contrast to this behaviour, we have found that the rates of the rapid phases, which are attributed to peripheral electrostatic events, reveal a particular dependence

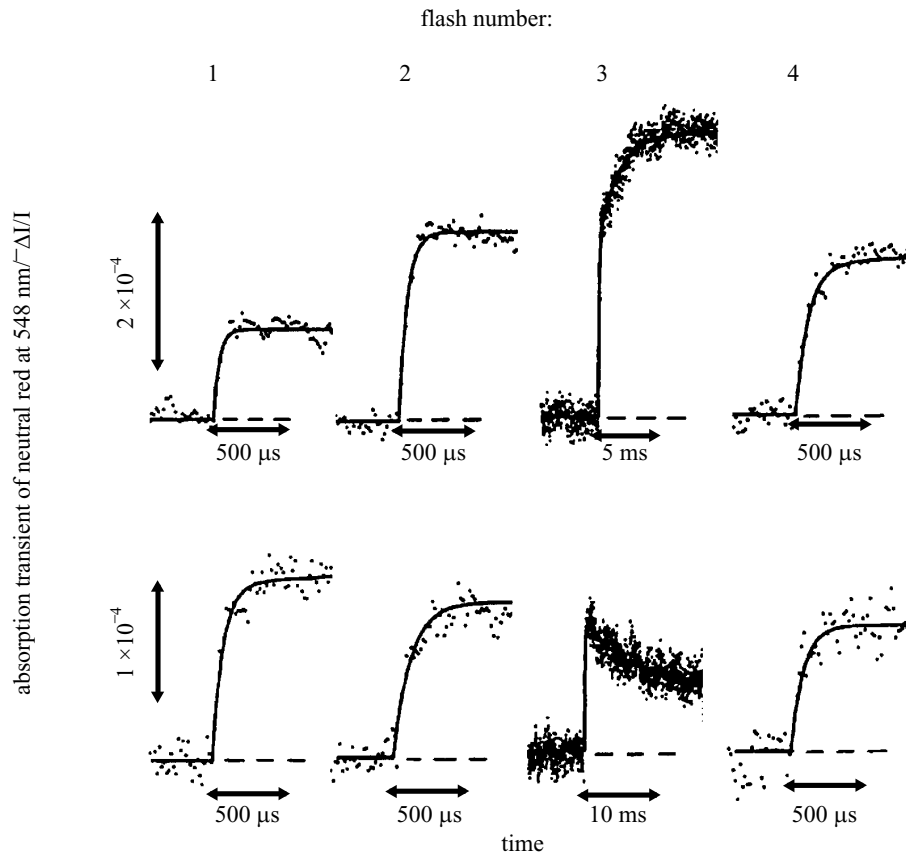
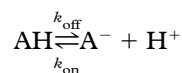


Figure 1. Time-resolved proton release after the first four flashes of a Q-switched Ruby laser given to unstacked, dark-adapted thylakoids from *pisum sativum* (Haumann & Junge 1994a). Proton release into the lumen of thylakoids was determined from absorption transients at 548 nm. They were recorded twice, plus and minus neutral red (45  $\mu\text{M}$ ), and in the presence of bovine serum albumin as impermeant buffer of pH transients in the external phase (Ausländer & Junge 1975; Junge *et al.* 1979). Note the different time-scale at the third flash, which causes mainly the oxygen-evolving transition  $\text{S}_3 \Rightarrow \text{S}_4 \rightarrow \text{S}_0$ . The half-rise times and the extents (in parentheses) resulting from a bi-exponential fit (lines) to the data (points) upon the third flash are in (a) pH 7.4: 40  $\mu\text{s}$  (0.9  $\text{H}^+$ ) and 1200  $\mu\text{s}$  (0.6  $\text{H}^+$ ), and (b) pH 6.3: 70  $\mu\text{s}$  (1.0  $\text{H}^+$ ) and 5000  $\mu\text{s}$  ( $-0.5 \text{H}^+$ ), respectively. The extents were normalized to the extent of proton release under repetitive excitation (1  $\text{H}^+$ ). For details see Haumann & Junge (1994a).

on the dye concentration and on the pH that is expected for proton transfer from an immediately activated source to a sink (the indicator dye). The mentioned 'immediate' activation is the electron abstraction from  $\text{Y}_Z$  by  $\text{P}_{680}^+$  in some 30–300 ns. Figure 3 illustrates the observed behaviour with thylakoids using neutral red as amphiphilic pH indicator (data in figure 3c) and with isolated PSII core particles using two hydrophilic pH indicators (data in figure 3d). As figure 3c shows with thylakoids the rate rises with increasing concentration of neutral red (Haumann & Junge 1994b). Such a behaviour is indicative for a bi-molecular collision involving neutral red. It is not unexpected as neutral red, an amphiphilic dye that is adsorbed at the membrane surface, will have an effective concentration in the thylakoid lumen at least a 1000-fold higher than in the bulk (Hong & Junge 1983; Junge *et al.* 1986). In contrast to thylakoid membranes, the rate of proton release from solubilized PSII core particles is independent of the concentration of added hydrophilic pH indicators. However it rises at a lower pH (data in figure 3d) (Bögershausen & Junge 1995). Such a behaviour is expected under two conditions: (i) the response is dominated by peripheral amino acids whose p*K* is in the range of the given pH, and (ii) the spontaneous protolysis of

these surface groups is followed by proton uptake by the hydrophilic indicator dye. The overall rate of the sequential reaction is limited by the rate of protolysis into the bulk.

The protolysis of an acidic group, A, at the surface is induced when its p*K* is acid shifted, e.g. by electrostatic interaction with a positive charge in the protein. The following reaction:



has a dissociation constant,  $K = k_{\text{off}}/k_{\text{on}}$ .

If the acid is directly in contact with bulk water, the on-rate is diffusion controlled ( $10^{10}$  to  $10^{11} \text{ M}^{-1} \text{ s}^{-1}$ ), and the off-rate is strictly p*K* controlled:  $k_{\text{off}} = k_{\text{on}} \times 10^{-\text{p}K}$  (see Eigen 1963; Gutman & Nachliel 1995) where  $\text{p}K = \log[\text{H}^+]$ . If the acid group is embedded in the protein, the relationship  $k_{\text{off}} = k_{\text{on}} \times 10^{-\text{p}K}$  still holds, but as the diffusion control of the on-reaction is lost, the p*K* control of  $k_{\text{off}}$  remains only broadly valid (so called free-energy relationship in elementary kinetics). Following this notion, we have interpreted the pH dependence of proton release in figure 3d as a p*K* dependence involving a set of groups with different p*K*s.

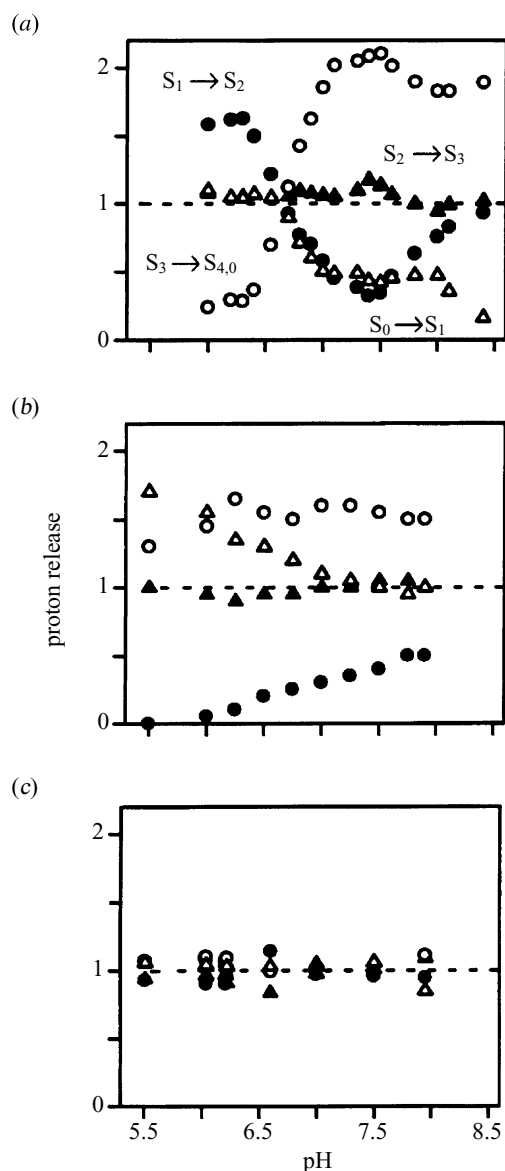


Figure 2. The relative extent of proton release attributable to the four sequential steps of the catalytic centre of water oxidation, namely  $S_0 \Rightarrow S_1$  (open triangles),  $S_1 \Rightarrow S_2$  (filled circles),  $S_2 \Rightarrow S_3$  (filled triangles) and  $S_3 \Rightarrow S_4 \rightarrow S_0$  (open circles). The extent was normalized to the extent under repetitive excitation ( $1H^+$ ). The original pattern of proton release (as in figure 1) was deconvoluted to yield the pattern over the steps under consideration of the  $S_0/S_1$  distribution in the dark and of double hits and misses. Three types of oxygen materials were used, (a) unstacked thylakoids (Haumann & Junge 1994a), (b) BBY membranes (Rappaport & Lavergne 1991), and (c) PSII core particles (Lübbers *et al.* 1993). For details, see the above references.

In summary, the proton transfer from the donor side of PSII to a given dye can be very fast under some conditions, or by two orders of magnitude slower under other conditions. The most rapid transients of proton transfer to the respective indicator dye, for example, with a half-rise time of 12  $\mu s$ , have been observed at a high concentration of neutral red in membranes (Haumann & Junge 1994a) or at acid pH to bromocresol purple with core particles (Bögershausen & Junge 1995). These rapid reactions imply a triggering at the level of oxidized  $Y_Z$ , or even  $P_{680}^+$ , so that proton release precedes the electron transfer

from the Mn cluster to  $Y_Z^{ox}$ . The slow proton release into the bulk (in core particles at alkaline pH), however, implies that the electrostatically induced release into the bulk of protons from the donor side may range into milliseconds without impairing the progress of oxygen evolution. It is noteworthy that we have not found any influence of the large variability of the extents and the rates of proton release on the rates of electron transfer between Mn and  $Y_Z$  and  $P_{680}^+$  and on the oscillatory pattern of electrochromic absorption transients (as documented and discussed in Lübbers *et al.* (1993)).

#### 4. SUPER-STOICHIOMETRY OF ELECTROSTATICALLY TRIGGERED PROTON RELEASE IN RESPONSE TO THE DEPOSITION OF A POSITIVE CHARGE IN THE CATALYTIC CENTRE

The above interpretation of the data implies that the electrostatic response of peripheral acid residues to the univalent oxidation of the donor side of PSII ( $Y_Z$  plus Mn cluster) can yield a proton per electron stoichiometry greater than one, in particular in thylakoids, during the transition of  $S_1 \Rightarrow S_2$  and at an acidic pH (see figure 1b and above). This 'super-stoichiometry' is not immediately plausible, given the fact that one peripheral acid, AH, after its deprotonation to yield  $A^-$ , tends to suppress the deprotonation of a neighbouring acid, BH, and consequently the formation of the doubly deprotonated pair  $A^-B^-$ .

A Monte Carlo treatment of electrostatically triggered proton uptake or release has been presented for the bacterial reaction centre (Beroza *et al.* 1991, 1995). It has been based on a high-resolution structure and involves very many acid or base residues. In the case of PSII, a rigorous treatment of the electrostatics, of proton release at the periphery of PSII, and of local electrochromic transients that are ascribed to the inner chlorophylls has to await the assignment of amino acids and the orientation of the cyclopentanone rings of the innermost chlorophyll molecules, both of which are not available in the structure at a 3.8 Å resolution (Zouni *et al.* 2001). Even if the amino acids were assigned, their protonation states and pKs will not be obvious without knowing their involvement in hydrogen bonding with neighbouring residues or crystallographically visible and invisible intra-protein water. It is for this reason that the following considerations are restricted to the question of whether or not it is possible, in principle, to obtain a  $H^+/e^-$ -super-stoichiometry by electrostatic interactions between a charge in the protein and peripheral acids. As a simple model lacking any structural detail, we consider the luminal surface of the thylakoid membrane as an infinite plane separating two homogeneous and infinitely extending phases, a conducting one, the thylakoid lumen and a non-conducting one, the membrane core. The presence of salts in the lumen and the very rapid lateral relaxation of field inhomogeneities along the luminal surface justifies the approximation of the lumen by a phase of 'infinite' conductivity. The globular structure of the PSII with its shielding extrinsic proteins that protrude from the membrane implies a pretty curved surface between the dielectric and the conducting phase, but not a flat one. The assumption of a flat geometry in the above simple model is just another

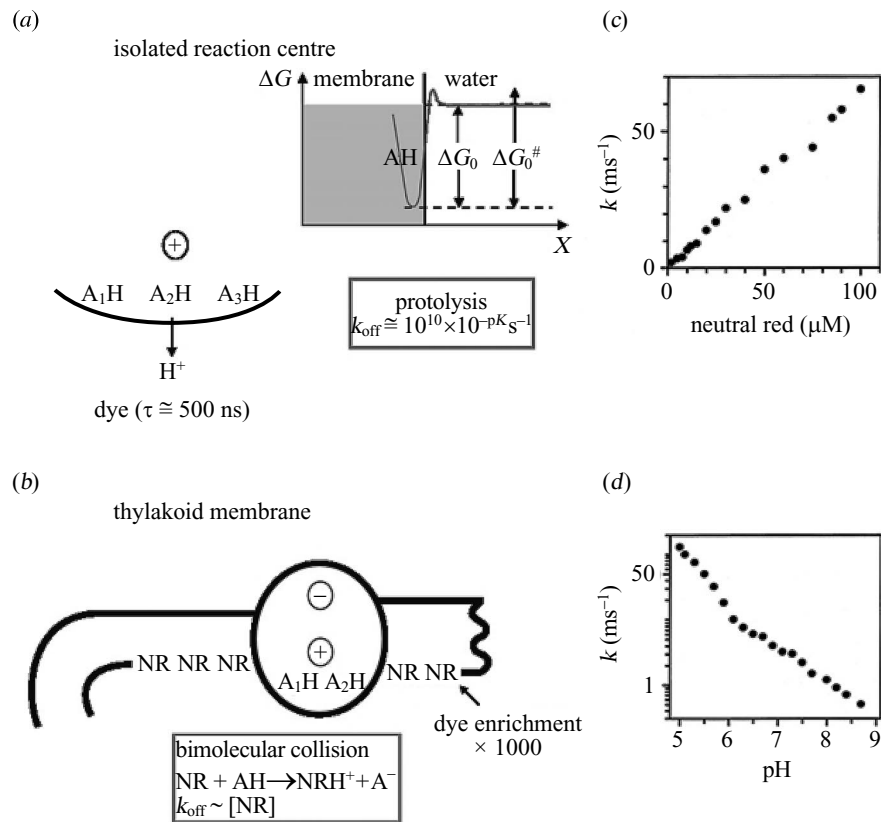
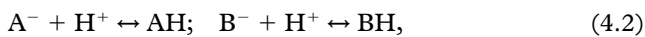


Figure 3. Different mechanisms of proton transfer to an indicator dye in detergent solubilized PSII core particles (a) and thylakoids (b), causing different properties of the rate constant of proton release under repetitive excitation with a Q-switched Ruby laser. (c) The rate of proton release as a function of the concentration of neutral red in unstacked thylakoids (Haumann & Junge 1994a), and (d) as function of the pH in PSII core particles and recorded by bromocresol purple ( $5 < \text{pH} < 7.5$ ) and cresol red ( $7 < \text{pH} < 8.7$ ) (Bögershausen & Junge 1995). For details, see the above references.

approximation, which is justified as long as details on water channels and perhaps high-dielectric pockets in the peripheral proteins are not available. We assume that the ‘manganese cluster’, M, resides  $10 \text{ \AA}$  deep in the membrane and that there are only two acid groups, AH and BH, symmetrically located to M and embedded  $3 \text{ \AA}$  deep in the membrane as illustrated in figure 4. The respective coordinates in angstrom units are as follows:

$$M: (0, 0, 10); \quad A: (0, -6, 3); \quad B: (0, 6, 3). \quad (4.1)$$

Both acids undergo reversible protonation and deprotonation with relaxation times which are shorter than typical measuring intervals used to assay the extent of the deprotonation (for kinetic features, see above). Thus they are both at equilibrium according to



with dissociation constants  $K_A$  and  $K_B$ , respectively.

The value of the dissociation constant of B depends on the charge states of  $M/M^+$  and the respective other acid,  $A/A^-$ . As an example, if both are charged,  $M^+$  and  $A^-$ , the  $pK = -\log K$  of the acid BH deviates from the one when both partners are electroneutral,  $pK_B^0$ , as follows:

$$pK_B(M^+, A^-) = pK_B^0 - \frac{e_0}{2.3k_b T} (\Phi_{MB} + \Phi_{AB}), \quad (4.3)$$

where  $e_0$  denotes the electric unit charge,  $k_b$  denotes the Boltzmann,  $T$  represents the absolute temperature and  $\Phi_{MB}$  and  $\Phi_{AB}$  denote the contribution to the electric

potential at B as caused by  $M^+$  and  $A^-$ . A positive potential jump which is caused by  $M^+$  lowers  $pK_B$  and favours the deprotonation of BH, whereas the negative potential by  $A^-$  increases  $pK_B$  and tends to reduce the deprotonation.

Because the lumen is assumed to be ‘infinitely’ conducting, the potential jumps can be calculated by the method of image charges (as presented in standard textbooks on electrostatics). The potential at the position of B,  $\Phi_B$ , which is caused by a charge of magnitude  $a_M e_0$  at the position of M is given by

$$\Phi_{MB} = \frac{a_M e_0}{4\pi\epsilon\epsilon_0} \left\{ \frac{[(x_B - x_M)^2 + (y_B - y_M)^2 + (z_B - z_M)^2]^{-1/2}}{ - [(x_B - x_M)^2 + (y_B - y_M)^2 + (z_B + z_M)^2]^{-1/2}} \right\}, \quad (4.4)$$

where  $\epsilon$  and  $\epsilon_0$  denote the relative and absolute dielectric permittivity. This is the well-known Coulomb potential. The second term represents the contribution of the ‘image charge’, a fictive charge of opposite sign to the one on  $M^+$ , which is symmetrically located to M in the conducting phase. The image charge guarantees that the interface is equipotential. In the given example its coordinates are  $M': (0, 0, -10)$ .

With the above coordinates of A, B and M and their respective image charges, the potentials generated by  $M^+$  and  $A^-$  at position B as calculated by equation (4.4) are as follows:

$$\Phi_{MB} = +139 \text{ mV} \quad \Phi_{AB} = -32 \text{ mV}. \quad (4.5)$$

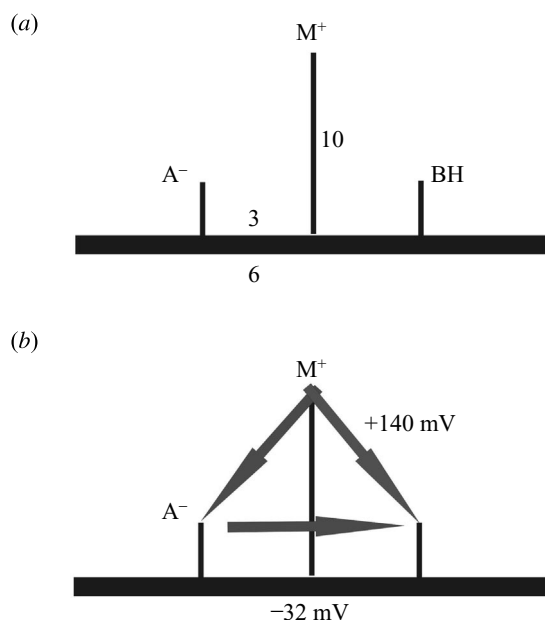


Figure 4. (a) Membrane; (b) buffer. Model for the electrostatic interactions between a charged carrier in a protein and two acids at the periphery which cause proton release into the bulk (Bohr effect). A photochemical reaction creates a positive charge at the position M which is embedded 10 Å deep in the protein. The protein is viewed as a homogeneous dielectric ( $\epsilon = 4$ ) facing a conducting bulk phase filled with conducting buffer. Two ‘peripheral’ acid groups, AH and BH<sub>2</sub>, which are embedded only 3 Å deep in the dielectric and 6 Å remote from the normal through M are subjected to a positive potential jump of +140 mV when M is charged to yield M<sup>+</sup>. If one of them is deprotonated, (A<sup>-</sup>, see bottom of diagram), it confers a potential of -32 mV to the other acid, BH. (For details see text).

This is illustrated in the bottom of figure 4.

They cause the following p*K* shifts of B (and likewise of A):

$$\Delta pK_{MA} = \Delta pK_{MB} = -2.3; \Delta pK_{BA} = \Delta pK_{AB} = +0.53. \quad (4.6)$$

Each (macro)state of M, namely M and M<sup>+</sup>, comprises four (micro)states of the acid- or base-couple A and B with *a priori* probabilities,  $w_1, w_2, w_3, w_4$ :

$$\text{AH BH } w_1 = (1 + 10^{\text{pH} - \text{p}K_A})^{-1} \times (1 + 10^{\text{pH} - \text{p}K_B})^{-1}, \quad (4.7a)$$

$$\text{A}^- \text{ BH } w_2 = (1 + 10^{\text{p}K_A - \text{pH}})^{-1} \times (1 + 10^{\text{pH} - \text{p}K_B})^{-1}, \quad (4.7b)$$

$$\text{AH B}^- w_3 = w_2, \quad (4.7c)$$

$$\text{A}^- \text{ B}^- w_4 = (1 + 10^{\text{p}K_A - \text{pH}})^{-1} \times (1 + 10^{\text{p}K_B - \text{pH}})^{-1}, \quad (4.7d)$$

where the respective figures of p*K*<sub>A</sub> and p*K*<sub>B</sub> differ depending on the charge state of the respective neighbours as given by equation (4.3). The respective p*K* shifts as calculated for the assumed arrangement of M, A and B are given by equation (4.6). If, for example, M is uncharged, p*K*<sub>A</sub> denotes p*K*<sub>A</sub><sup>0</sup> in equations (4.7a,b) and p*K*<sub>A</sub><sup>0</sup> + 0.53 in equations (4.7c,d). If, however, M is positively charged p*K*<sub>A</sub> denotes p*K*<sub>A</sub><sup>0</sup> - 2.3 in equations (4.7a,b) and p*K*<sub>A</sub><sup>0</sup> + 0.53 - 2.3 in equations (4.7c,d).

The extent of deprotonation,  $\Delta\text{H}^+$ , of the pair of inter-

acting acids is given by the weighted contributions of the microstates (equations (4.7)),

$$\Delta\text{H}^+ = (2w_2 + 2w_4)/(w_1 + 2w_2 + w_4). \quad (4.8)$$

Its magnitude varies depending on whether M is charged or uncharged. For simplicity we assume that the undisturbed p*K*s (M is uncharged) of both acids are equal, p*K*<sub>A</sub><sup>0</sup> = p*K*<sub>B</sub><sup>0</sup> = p*K*<sup>0</sup>, and further that the ambient pH = p*K*<sup>0</sup> - 1. The extent of the deprotonation per pair of acids, which is caused by the univalent up-charging of M to yield M<sup>+</sup>, then amounts to the following:

$$\begin{aligned} \Delta\Delta\text{H}^+ &= (\Delta\text{H}_{M^+}^+ - \Delta\text{H}_M^+) \\ &= (1.738 - 0.177) = 1.561. \end{aligned} \quad (4.9)$$

We found that only two acid residues at a reasonable spacing to the Mn cluster and between each other can produce a super-stoichiometry of proton release in response to the univalent up-charging of M. The extent of electrostatically driven proton release, as opposed to the one that is caused by the chemistry in the catalytic centre proper, depends on the number and the topology of peripheral amino acids relative to the centre, their original p*K*s, the ambient pH and the dielectric environment of these acids as given in principle by equations (4.3), (4.4) and (4.7). The simple electrostatic model presented above can, of course, be extended to incorporate the stromal bulk phase (this brings in further image charges), to account for globular protein structure and for the involvement of more acid residues. The formalism to treat such systems is standard in statistical thermodynamics. These extensions will not bear on the possibility, in principle, to obtain super-stoichiometries of proton release.

One simplifying assumption, implicit in the above considerations is, however, critical. We assume that the conversion of M to M<sup>+</sup> is irreversible, the appearance of the positive charge on M is not affected by the charge state of the acids. In other words, we neglect the electrostatic back-pressure of the deprotonated and negatively charged peripheral acids on the redox potentials of M and its reaction partner. This neglect appears adequate for a photochemical reaction with large driving force, but not necessarily if the driving force of the reaction M → M<sup>+</sup> is small. Under these conditions a network approach including the equilibrium M<sup>+</sup>/M is more appropriate.

One of us (M.H.) simulated the observed dramatic variation of proton release in thylakoids as function of the pH and its constancy in detergent solubilized core particles by similar reasoning as laid out for the simple model above and obtained a reasonable fit to the above-presented data with only three acid groups. The pH independence of local electrochromic shifts was also modelled. Because of the still existing freedom to choose the positions and orientation coordinates of the cofactors, the dielectrically weighted overall topology and the p*K*s of the interacting acid or base groups, such fits just serve illustrative purposes. That is why we refrained from presenting results. A rigorous treatment, as mentioned above, has to wait for a very much perfected structural model of PSII.

## 5. PROTON ROCKING BETWEEN Y<sub>2</sub> AND AN ACID OR BASE CLUSTER IN RESPONSE TO ITS OXIDO-REDUCTION

Rapidly rising light-induced absorption transients have been attributed to electrochromic bandshifts of chloro-

phyll *a* accompany the oxidation of  $Y_Z$  by  $P_{680}^+$  (Rappaport *et al.* 1994). The major portion decays with the typical time constant of the electron transfer from the Mn cluster to  $Y_Z^{\text{ox}}$ . Rappaport and colleagues also reported a smaller, more rapidly decaying component, which they attributed to an intra-protein proton transfer around  $Y_Z$ , a feature that we did not observe in our experiments despite sufficiently high time resolution (Haumann *et al.* 1994; Haumann & Junge 1996). The overall behaviour, rapid rise and slow partial decay of the electrochromic transient, with the latter following the reduction of  $Y_Z^{\text{ox}}$  by the Mn cluster, point to the lack of charge neutralization, e.g. by release into the bulk of the phenolic proton of  $Y_Z$  (Lavergne & Junge 1993; Haumann & Junge 1996). This view has been questioned by others (Hoganson & Babcock 1997) who have interpreted one specific proton per electron stoichiometry, namely 1 : 1 as found under some conditions in core particles (see figure 2c), as the unmasked release into the bulk of the proton from  $Y_Z^{\text{ox}}$  itself. The assumed electrochromic bandshifts have been re-interpreted as through-bond interactions between the tyrosine and the chlorophyll *a* of  $P_{680}$  (Tommos *et al.* 1998). It has therefore been claimed that  $Y_Z^{\text{ox}}$  is reprotonated from water molecules upon each reduction (Britt 1996; Tommos & Babcock 2000). This matter has not yet been rigorously settled. There is no proton release from  $Y_Z^{\text{ox}}$  proper, at least as long as the Mn cluster is in the  $S_1$  state. This is documented in figure 1 (see first transient at pH 7.4). Here, the net extent of proton release with a half-rise time of 12  $\mu\text{s}$  (i.e. before any reaction with the Mn cluster) is about 0.5 protons. If there had been the production of a chemical proton and its release from  $Y_Z$  into the bulk, a larger signal would have been observed. Admittedly, this evidence bears only on this particular redox transition, namely  $S_1 \Rightarrow S_2$ .

It has been work with severely modified, i.e. Mn- and/or Ca-depleted PSII core preparations that has provided indirect evidence for the absence of a deprotonation of  $Y_Z$  into the bulk during the other redox transitions even in the intact, i.e. oxygen-evolving PSII. This line of evidence is related to the susceptibility of a neighbouring base cluster around  $Y_Z$  to allow rapid electron transfer from  $Y_Z$  to  $P_{680}^+$ . If, as in Mn-depleted material at an acid pH, this cluster is saturated, the phenolic proton of the  $Y_Z$  tyrosine has to be ejected into the bulk before  $Y_Z$  can be oxidized by  $P_{680}^+$  in a then proton-controlled reaction (Ahlbrink *et al.* 1998; Diner *et al.* 1998; Hays *et al.* 1998, 1999; Mamedov *et al.* 1998; Haumann & Junge 1999a). Turning this argument around to oxygen-evolving PSII, where the electron transfer to  $P_{680}$  is very fast (30–300 ns), it implies proton rocking between  $Y_Z$  and the base cluster and its transient upcharging upon the oxidation of  $Y_Z$ .

The data favouring this notion, obtained with Mn-depleted PSII core particles, are given in figure 5. Whereas the half-rise of the electron transfer to  $P_{680}^+$  in the intact material ranges between 30 and 300 ns, there is a biphasic rise in Mn-depleted centres. A fast phase rises in *ca.* 1  $\mu\text{s}$ . Its rate is almost independent of the pH (see figure 5a). A slower phase rises in 10–100 ms and the rate decreases at lower pH. Their summed extent is constant (see figure 5b), if one corrects for charge pair recombination (see figure 5c). They are mutually inter-convertible as a function of the pH, and the transition between them titrates

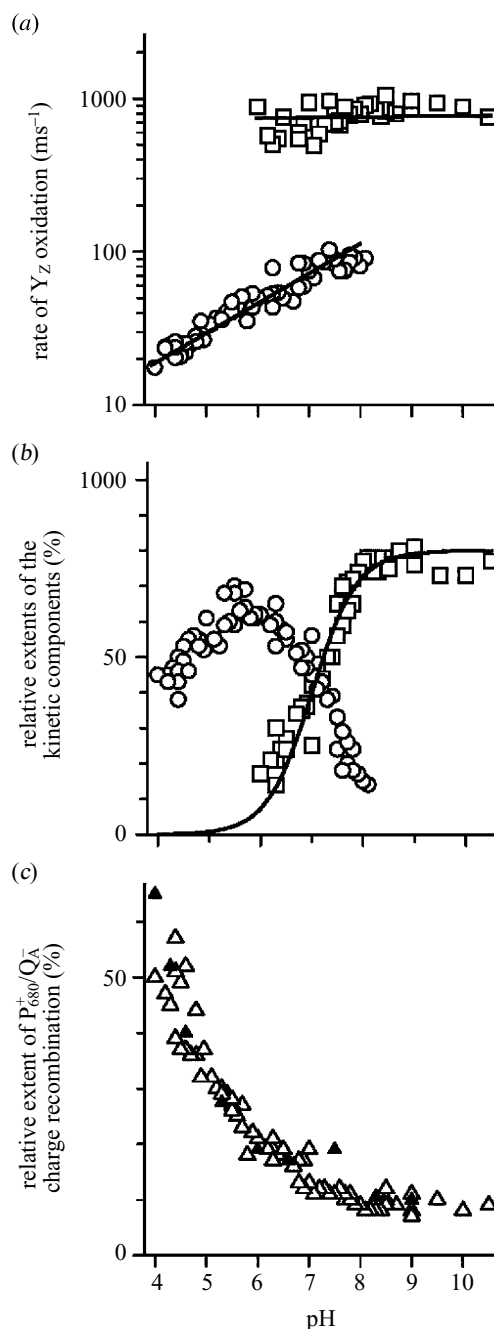


Figure 5. The rates and extents of the kinetic components of the reduction of  $P_{680}^+$  in inactive PSII core particles as a function of the pH, according to a fit with three exponentials (Ahlbrink *et al.* 1998). (a) The rates of the two faster kinetic components. The rate of the fastest component,  $k_f$  (squares) is pH independent (line), whereas the rate of the slower one,  $k_s$  (circles) decreases with the pH (line). (b) The relative extents of the two kinetic components ( $k_f$ , squares;  $k_s$ , circles) from (a). The extents of  $k_f$  are described by a single titration with a  $pK$  of 7 (line). (c) The sum of the extents of the very slow components ( $k_v$ ) as function of the pH. Open triangles, data from absorption transients at 827 nm which reflect the oxidoreduction of  $P_{680}$ ; solid triangles, data from transients at 320 nm which reflect the oxidoreduction of  $Q_A$ . For details see Ahlbrink *et al.* (1998).

with a  $pK$  of *ca.* 7 (figure 5b). The rise time of the nanosecond components in fully functional, oxygen-evolving core particles and of the microsecond component in Mn-depleted material are both pH independent (between pH

Table 1. Half times of electron transfer reactions in cells and PSII core particles from different mutants compared with wild-type. (Mean values with standard deviations. For details, see Clausen *et al.* (2001). +/-G, presence or absence of glycerol; *n*, number of measurements.)

| reaction                                     | wild-type  | E189Q  | E189K  | E189R  |
|--|--|--|--|--|
| S <sub>1</sub> ⇒ S <sub>2</sub>              | 46 ± 5 μs; <i>n</i> = 190  | 55 ± 40 μs <sup>b</sup> ; <i>n</i> = 20                            | 44 ± 7 μs; <i>n</i> = 80                           | 41 ± 7 μs; <i>n</i> = 90                         |
| S <sub>2</sub> ⇒ S <sub>3</sub>              | 90 μs; <i>n</i> = 190  | 90 ± 50 μs <sup>b</sup> ; <i>n</i> = 20                            | 99 ± 7 μs; <i>n</i> = 80                           | 96 ± 7 μs; <i>n</i> = 90                         |
| S <sub>3</sub> ⇒ S <sub>0</sub> (cores, -G)  | 4.5 ± 0.4 ms <sup>a,b</sup> ; <i>n</i> = 100                         | 4.7 ± 0.5 ms <sup>b,c</sup> ; <i>n</i> = 80                        | —  | —  |
| S <sub>3</sub> ⇒ S <sub>0</sub> (cores, +G)  | 1.5 ± 0.1 ms <sup>a,b</sup>  | —  | —  | —  |
| S <sub>3</sub> ⇒ S <sub>0</sub> (cells)      | 1.5 ± 0.5 ms <sup>b</sup> ; <i>n</i> = 5                             | 1.5 ± 0.5 ms <sup>b</sup> ; <i>n</i> = 5                           | —  | —  |
| reduction of P <sub>680</sub> <sup>+</sup> : |  |  |  |  |
| oxygen-evolving PSII                         | 30 ± 3 ns (36%)  | 38 ± 2 ns <sup>b</sup> (24%)                                       | 27 ± 0 ns (48%)                                    | 30 ± 1 ns (41%)                                  |
|  | 253 ± 21 ns (20%)<br>> 2 μs (45%);<br><i>n</i> = 1950                | 259 ± 10 ns <sup>b</sup> (21%);<br>> 2 μs (55%);<br><i>n</i> = 800 | 251 ± 6 ns (21%);<br>> 2 μs (31%); <i>n</i> = 1800 | 304 ± 21 ns (24%); > 2 μs (35%); <i>n</i> = 1750 |
| Mn-depleted PSII                             | 780 ± 10 μs <sup>b,c</sup> (87%);<br>> 2 ms (13%);<br><i>n</i> = 300 | 650 ± 20 μs <sup>b,c</sup> (96%);<br>> 2 ms (4%); <i>n</i> = 150   | —  | —  |

<sup>a</sup> Data from Haumann *et al.* (1997b).

<sup>b</sup> Error calculated from the fit routine.

<sup>c</sup> Core particles prepared after Hundelt *et al.* (1998).

5.5 and 7.5) (Meyer *et al.* 1989), nearly insensitive to H<sub>2</sub>O/D<sub>2</sub>O isotopic substitution (Haumann *et al.* 1997a; Ahlbrink *et al.* 1998), and they reveal a low activation energy. In contrast to the former, the rate of the slow component in Mn-depleted material decreases with decreasing pH, the kinetic H/D isotope effect is 2.5 and the activation energy is high (0.3 eV). It is obvious that the reaction between Y<sub>Z</sub> and P<sub>680</sub><sup>+</sup> switches at pH 7 from electron- (fast) to proton-controlled (slow) electron transfer (Ahlbrink *et al.* 1998).

These observations have been interpreted to indicate that, in the intact system, the rapid reduction of P<sub>680</sub><sup>+</sup> requires the presence of a receptive base cluster around Y<sub>Z</sub>. If this cluster is protonated as at acid pH (at pH < 7 in Mn-depleted centres, and possibly at pH < 4.5 in intact ones), the electron transfer between Y<sub>Z</sub> and P<sub>680</sub><sup>+</sup> is kinetically controlled by proton transfer (see the H/D-isotope effect in Ahlbrink *et al.* (1998)). Only under these conditions, the normal electrochromic transients of chlorophyll *a* vanished (see figure 9 in Ahlbrink *et al.* 1998) as if proton release into the bulk from the vicinity of Y<sub>Z</sub> was then the prerequisite of the electron transfer to P<sub>680</sub><sup>+</sup>. Under these conditions, we observed proton release into the bulk with a similar rate as one of the electron transfers. These phenomena have been understood in terms of a rise of the midpoint potential of Y<sub>Z</sub><sup>ox</sup>/Y<sub>Z</sub> by 0.1 V when the base cluster is protonated at acid pH (Ahlbrink *et al.* 1998). This notion is compatible with the reported difference of the midpoint potentials of Y<sub>Z</sub> and P<sub>680</sub> in Mn-depleted material, namely 0.1 V at pH 6.5 (Metz *et al.* 1989; Mulikidjanian *et al.* 1996). In essence, these studies have revealed that the phenolic proton of Y<sub>Z</sub> upon oxidation can be released into and then detected in the bulk. This occurs, however, only in Mn-depleted material at acid pH. Under other conditions, and most importantly in fully functional PSII, the phenolic proton remains in the vicinity of Y<sub>Z</sub> and this may be one construction element to increase the redox potential of Y<sub>Z</sub> relative to the Mn cluster (Ahlbrink *et al.* 1998). Models of the catalytic events where it is assumed that Y<sub>Z</sub> is deprotonated into the bulk

phase upon every transition in order to function as a hydrogen acceptor for water (Tommos & Babcock 1998, 2000) are difficult to reconcile with this notion.

It is noteworthy that the peculiar kinetic behaviour of the electron transfer from Y<sub>Z</sub> to P<sub>680</sub><sup>+</sup> in Mn-depleted PSII (figure 5a, Ahlbrink *et al.* 1998) has been almost perfectly mirrored in a synthetic ruthenium-pyridyl-tyrosine construct (see Sjödin *et al.* 2000, 2002) where the pH dependence of the slow phase has been interpreted as concerted proton–electron transfer.

Two independent techniques, namely with thylakoids, the electrochromic transients of intrinsic carotenoids (Junge & Witt 1968) and with PSII liposomes, an electrochromic technique (Drachev *et al.* 1981), have been used to determine the transmembrane electrogenicity of the electron and proton transfer in PSII (Haumann *et al.* 1997c). The results, agreeing with each other, were as follows: taking the electrogenicity of the electron transfer from Y<sub>Z</sub> to Q as 100%, the step from Y<sub>Z</sub> to P<sub>680</sub><sup>+</sup> accounts for 15%, and from the Mn cluster to Y<sub>Z</sub> for *ca.* 3%. The electrogenicity of proton transfer out of the catalytic centre into the bulk (e.g. the release of chemically produced protons during S<sub>3</sub> ⇒ S<sub>0</sub>) is variable, it ranges between 2 and 10%. These observations are fully in line with the above notion of the redistribution of protons between Y<sub>Z</sub>, its base cluster, peripheral acids and the bulk (for data and a detailed discussion see Haumann *et al.* (1997b,c)). The figures for the relative electrogenicity are related to the projected positions of the cofactors along the normal membrane, through weighting by dielectric permittivity, ε, as distance times ε appear in the Coulomb energy (see equation (4.4)).

## 6. A SPECIAL ROLE OF D1-HIS190 IN THE ACID OR BASE CLUSTER AROUND Y<sub>Z</sub>?

Mutant analyses of PSII have indicated that D1-His190 is one essential hydrogen-bonded partner of Y<sub>Z</sub>. One surprising and convincing result has been the rescue by soluble weak acids of the rapid electron transfer from Y<sub>Z</sub> to



$P_{680}^+$  in a mutant where D1-His190 is replaced by neutral amino acids (Hays *et al.* 1998, 1999). It has been speculated that D1-Glu189, the direct neighbour of D1-His190, is another member of the hydrogen-bonded base cluster around  $Y_Z$  (Debus *et al.* 2000). We checked this suggestion by measuring the rates of electron transfer from the Mn cluster to  $Y_Z^{\text{ox}}$  (in  $\mu\text{s}$ ) and from  $Y_Z$  to  $P_{680}^+$  (in ns) and found no difference between the wild-type and E189Q, E189K and E189R (see table 1; Clausen *et al.* 2001). This result is surprising because one expects some effect because of different electrostatic properties of glutamic acid, glutamine, arginine and lysine. The lack of any effect of an acid, neutral or basic residue at position D1-E189, then implies that it is embedded either in a strongly hydrophobic environment (all residues are forcedly electro-neutral) or in high dielectric (the charge is fully shielded). Otherwise, the absence of any electrostatic effect of the D1-E189 mutants could be explained by a distance between  $Y_Z$  and D1-H190, D1-E189 which is much greater than so far supposed. The advanced crystal structure of PSII (see Fromme *et al.* 2002) indicates that the D1-His-190 may indeed be more than 10 Å away from the position of  $Y_Z$ .

## 7. SUMMARY

We discriminated the chemical production of protons at the donor side of PSII from electrostatic Bohr effects, as well as proton rocking between cofactors from proton release into the bulk. The results are compatible with the notion that the catalytic centre is positively up-charged during the transition  $S_1 \Rightarrow S_2$  and electrostatically relaxed during  $S_3 \Rightarrow S_4 \rightarrow S_0$ , as inferred from previous studies on electrochromic transients. Protons are chemically produced and then released into the bulk phase at the luminal side of PSII at least during transitions  $S_2 \Rightarrow S_3$  and  $S_3 \Rightarrow S_4 \rightarrow S_0$ . This conforms with the notion that the oxidizing equivalent during transitions  $S_2 \Rightarrow S_3$  is not stored on Mn proper but rather on some ligand (see Yachandra 2002). The phenolic proton of  $Y_Z$  does not seem to be released into the bulk but rather rocking between  $Y_Z$  and a base cluster in its vicinity. The receptivity of this cluster is a prerequisite for the rapid electron transfer from  $Y_Z$  to  $P_{680}^+$ . If the cluster is pre-protonated, the electron transfer is slow and proton controlled (see Sjödin *et al.* (2002), for a similar behaviour in a synthetic model system). D1-His190 the proposed candidate for the hydrogen-bonded partner of  $Y_Z$ , seems to be more remote from  $Y_Z$  than previously thought, because electrostatically differing mutations at the neighbouring position, D1-Glu189, have little if any influence on the electron transport between the Mn cluster,  $Y_Z$  and  $P_{680}$ . These features of proton retention and proton release are probable key elements in the remarkable tuning of the four stepped reaction cycle to one and the same energy supply.

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### Discussion

C. Tommos (*Department of Biochemistry and Biophysics, Stockholm University, Stockholm, Sweden*). I have two questions. Is X a cofactor? Have you undertaken a time-resolved study for H<sup>+</sup> release from PSII core particles that does not involve repetitive flashes?

W. Junge. We found that the transition S<sub>2</sub> → S<sub>3</sub> caused proton release with the same time constant as of the electron transfer from Mn to Y<sub>Z</sub><sup>ox</sup>, and we named the unknown chemical source X. As Mn proper cannot release a proton upon oxidation, we claimed that a cofactor of Mn (bridging oxygen, bound water) was involved. A time-resolved study of proton release in dark-adapted samples has not yet, to our knowledge, been carried out to the same time-resolution as published for experiments with repetitive excitation.

S. Styring (*Department of Biochemistry, Lund University, Lund, Sweden*). I wish to make a comment regarding TyrZ oxidation in Mn-intact and Mn-depleted PSII and how His190 might be involved. In Mn-depleted PSII, TyrZ gives off a proton on oxidation and, from Brudvig's work, we know that TyrZ is not oxidized below 200 K. Thus, there must exist a base that picks up the proton. The problem is that there are very few alternative bases to His190. In Mn-intact PSII, we observe TyrZ oxidation at 5 K, thus there must exist a pre-formed H-bond or TyrZ is a tyrosinate.

W. Junge. Indeed, what you said was what we had in mind.

P. Rich (*Department of Biology, University College London, London, UK*). I would like to ask a general question. You have suggested purely from electrostatics that you can reduce a component that is interacting equally with two

protonateable groups and get more than one proton uptake. I suspect that is incorrect. Could you explain your logic? Second, are there any chemicals that undergo more than one protonation for one charge event governed purely by electrostatics?

W. Junge. The electrostatic calculus tells you the following. If you deposit an electric charge in a dielectric that faces a conductor, the electric potential jump experienced by peripheral acids in the dielectric can be large enough so that several acids undergo deprotonation, even when considering their repulsive interaction. The presumption is that the triggering charge is irreversibly deposited in the dielectric. This situation may be approximated in photosynthetic reaction centres, but not necessarily met in cytochrome oxidase, where the driving force of the primary electron transfer is smaller than supplied by a photochemical reaction.

B. Diner (*Central Research and Development Department, EI DuPont de Nemours and Company, Wilmington, DE, USA*). There are experiments which show that  $H^+$  release at pH 5 and below does not occur with non-oxygen-evolving PSII preparations. Therefore, under these conditions the protons must remain within the membrane. How does this reconcile with your electrostatic model?

W. Junge. The extent of electrostatic proton release depends on the availability near the surface of acidic groups with a  $pK$  in the appropriate range, for example,  $pK = pH + 1$ .

C. Zhang (*Department of Biochemistry, Lund University, Lund, Sweden*). I am interested in the data you report. You suggested that the positive charge is on 'X', but this is not in the Mn cluster. Why? I think that if the positive charge is delocalized on the Mn cluster, we can explain why the oxidation of  $Y_Z$  is dependent on the state of the metal centre. However, if the positive charge is delocalized on the ligands of the Mn cluster, does that not mean that the positive charge could also be delocalized on the Mn cluster?

W. Junge. It has been reported (see Yachandra 2002) that manganese itself undergoes a valency shift during the transition  $S_1 \rightarrow S_2$ . This is less certain for  $S_2 \rightarrow S_3$ . Here, the electron hole may reside on a ligand rather than on manganese proper. Delocalization of the electron hole over manganese and its ligands may prevail in any transition, with variations of the distribution between manganese and ligand. What we said relating to proton release was simply the following: during  $S_2 \rightarrow S_3$  there is proton release from manganese plus ligands and the proton originates from a component X (a ligand) which lost electron density during this particular transition.

## GLOSSARY

PSII: photosystem II